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Applications of Cathelicidin

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Localization, Concentration, and Antimicrobial Activity of Human Cathelicidin LL-37 from H1299 Human Lung Carcinoma Cells to be applied to Dermal Wound Healing

A Major Qualifying Project

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Abstract

Wound infection is a medical problem that does not receive immense attention. Common therapies for dermal infection include disinfectants, antiseptics, and finally antibiotics if the infection becomes systemic (Siddiqui & Bernstein, 2010). Wounds that persist for greater than six weeks are considered chronic and require secondary therapies such as skin grafts to aid in the healing process (Macri, Silverstein, & Clark, 2007). Extracellular matrices (ECM) have been shown to improve wound healing however they lack properties that can combat infection. In 2011, a recombinant form of the antimicrobial polypeptide, LL-37 Cathelicidin, was designed to be used as an antimicrobial property in ECMs for dermal wound healing. The recombinant peptide designed for this project has an affinity to bind to the either the collagen or fibronectin matrix of dermal fibroblasts to be used as the antimicrobial property of the ECM. However, previous research has only verified the recombinant construct through sequence analysis and preliminary expression in H1299 human lung carcinoma cells. This research project investigated expression and secretion of the recombinant Cathelicidin. Stably transfected H1299 cell line expressing recombinant LL-37 was produced and initial testing was conducted to concentrate the peptide, test the peptide's antimicrobial activity against bacteria commonly found on skin, and carry out initial attachment affinity testing to collagen and fibronectin surfaces. Incorporation of this peptide into a cell-derived ECM is beneficial as there is less bacterial resistance to antimicrobial peptides.

Table of Contents

Table of Figures

Table of Tables

Introduction

Dermal Wound Infection

Skin is the body's first defense against the elements that are harmful to the immune system. When compromised, the body is exposed to infection that can cause systemic problems. Physical trauma, burns, surgery, and pathologically colonized ulcers are the most common ways for the dermal barrier to be affected (Macri and Clark 2009). Bacterial wound infections are the 28th most commonly diagnosed condition in hospital patients (Stulberg, Penrod, & Blatny, 2002). 20% of dermal wounds will become chronic, meaning that the healing process of a wound has exceeded a six-week period (Macri et al., 2007). Though each stage of the wound healing reconstruction process is not fully understood, it is believed that bacterial manifestation is the initial cause of chronic wounds (Siddiqui & Bernstein, 2010).

The most common bacteria found in dermal wounds are *Staphylococcus aureus, Enterococcus faecalis,* and *Pseudomonas aeruginosa (Gjødsbøl et al., 2006).* Due to the fact that immunological cells are not necessary in the later stage of wound reconstruction, in chronic wounds, bacteria are able to proliferate producing a persistent infection (Wilgus, 2008). The bacterial load known to hinder the healing of a wound is 10^5 organisms/g of tissue. Infection is usually combated by use of topical disinfectants, topical antiseptics, and finally antibiotics if the infection spreads to a systemic proportion. However, once a wound has entered in to the chronic stage topical antiseptics and disinfectants can no longer be used and a secondary therapy must be employed (Siddiqui & Bernstein, 2010).

Extracellular Matrices

The use of extracellular matrices (ECMs) in the field of tissue engineering (TE) is one of great promise. The extracellular matrix behaves essentially as a scaffold for new tissue to develop, allowing regrowth of damaged tissue (Silver, 2006). The goal of TE is to use these new technologies and techniques in order to assist patients with previously difficult to treat wounds (Zhong, 2010). In order to achieve more success, ECMs have to not only match the physical and chemical characteristics of the tissue they're grafted into, but also have to provide defense against infection and the correct chemical and mechanical signals to encourage new cell growth (Zhong, 2010).

The extracellular matrices themselves are typically created from natural polymers, such as collagen and fibronectin, or a variety of similar artificial polymers such as poly-l-lactic acid (PLLA) or polyglycolic acid (PGA) (O'Brien, 2011). Produced in the right manner, these and many other materials can exhibit the necessary properties for an ECM in the human body. Different materials have different attributes, which must be analyzed carefully when choosing for healing. Synthetic materials, for example, are easy to create and have very predictable properties; however, their interactions with biological tissue can cause significant scarring in the healed wound (Ruszczak, 2003).

Collagen is generally accepted as the ideal material out of which to construct ECMs. Collagen sponges made of animal tissues stripped of natural cells contain an excellent combination of the correct structure, and certain specific sequences of the collagen fibers can enhance the cellular growth more so than other constructs (Ruszczak, 2003). They also have been shown to have very low rejection rates, and can be quickly integrated into the body (Ruszczak, 2003). In general, it has been shown that use of collagen as an ECM material is more effective than many artificial polymers (Horch 1998).

Treatment Solution

The secondary therapy being proposed for the treatment of chronic wounds is a cell derived ECM with antimicrobial properties as shown in Figure 1. Treatment over time with antibiotics can cause bacterial resistant strains to form, but there is less bacterial resistance to antimicrobial polypeptides (AMPs) due to the mechanism with which they react with bacteria (Wang, 2008). This reduced resistance makes AMPs a beneficial addition to an ECM. The peptides also need to attach to an ECM to stay for the duration of therapy. Attachment to an ECM would allow for the wound to be washed and undergo the natural healing process without the loss of the antimicrobial property. The overall goal would be to create a cell derived ECM with cells that secrete the peptide at a therapeutic concentration that is still to be determined.

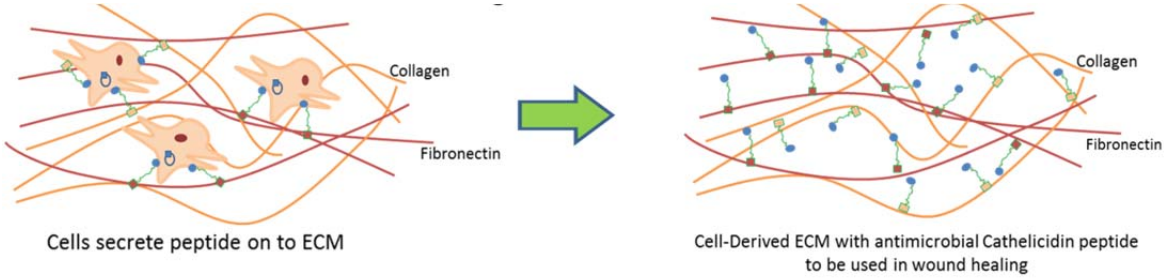

Figure 1: Overall research goal of producing a cell derived ECM producing an AMP

Cathelicidin and Cathelicidin Construct

AMPs are an innate part of the immunological cascade and can be found in mammals in the form of defensins and cathelicidins (Wang, 2008). The only human cathelicidin AMP to be isolated is the LL-37 cathelicidin peptide (Wang, 2008). The LL-37 peptide consists of a precursor region hCAP-18, followed by the cathelin like domain that is present in all N-terminus ends of cathelicidins, and the AMP specific LL-37 region consisting of 37 amino acids (Li, Li, Han, Miller, & Wang, 2006). Figure 2 shows the amino acid sequence for these regions.

MKTQRDGHSLGRWSLVLLLLGLVMPLAII AQVLSYKEAVLRAI

DGINQRSSDANLYRLLDLDPRPTMDGDPDTPKPVSFTVKETV

CPRTTQQSPEDCDFKKDGLVKRCMGTVTLNQARGSFDISCDK

DNKRFALLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

Figure 2: Protein Data Bank sequencing for hCAP-18 (green), Cathelin-like domain (blue), and LL-37 sequence (red)

LL-37 has been found in beta cells, monocytes, mast cells, immature neutrophils and most importantly epithelia skin cells specifically keratinocytes (Dürr, Sudheendra, & Ramamoorthy, 2006). Figure 3 is an image of the LL-37 construct without the precursor hCAP-18 and the cathelin domain. LL-37 is located on the surface of the lipid bilayer in cells of the human body. It is an amphipathic alpha helix and is believed to target bacteria through cationic interactions to the anionic surface of bacteria (Wang, 2008). However, the exact mechanism of antimicrobial

action is still not understood as 3D structures of the intact LL-37 bound to bacteria have never been produced (Wang, 2008).

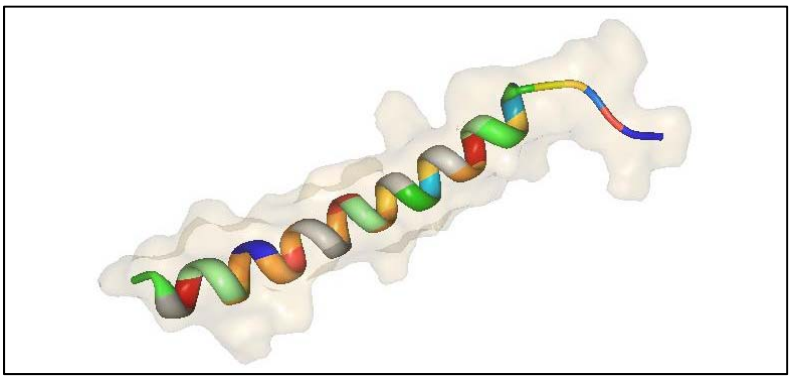

Figure 3: LL-37 amino acid sequence structure

Segmentation experiments have allowed for the antimicrobial activity of the LL-37 to be discovered. The residue KR-12 spanning amino acids 18-29 of the LL-37 holds the antimicrobial property for the antimicrobial polypeptide (Wang, 2008). Levels of LL-37 have been shown to increase after wound infliction to the skin barrier. Furthermore, potency of the AMP is affective to an array of bacteria, both gram negative and gram positive, leaving human cells unharmed (Dürr et al., 2006). These properties of the Cathelicidin LL-37 AMP make it a desirable addition to an ECM for therapeutic use in treatment of wound infection and regeneration.

In order to incorporate the LL-37 AMP in to an ECM, a recombinant was created to either anchor to a fibronectin or collagen surface. The fibronectin and collagen domains were chosen due to good affinity for their respective surfaces. Furthermore, the flag-tag peptide was inserted before the binding domain at the C-terminus of cathelicidin as to not hinder steric activity of the LL-37 AMP. Figure 4 shows the recombinant cathelicidin LL-37 structure that will be used in experimentation in this research (Prifti 2012).

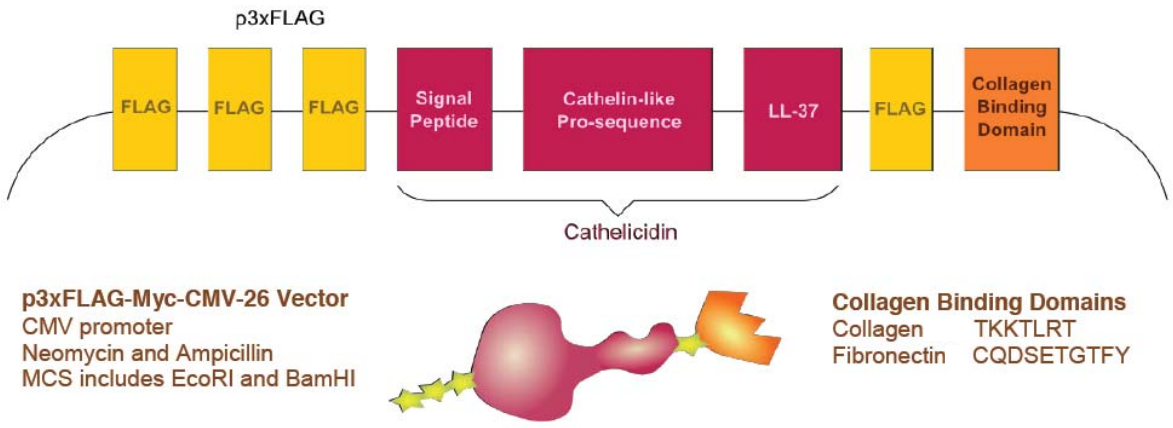

Figure 4: Gene design of recombinant collagen-binding cathelicidin insert (Prifti 2012)

Previous Data from 2011 Study

In 2011, the 510bp Cathelicidin gene was isolated from pANT7 cGST. It was then ligated into the pGEM vector to modify the gene with either a collagen or fibronectin binding domain (BD) (Col-BD or Fib-BD). This insert was transformed in *E. coli* and were selected and then sent for Sanger sequencing. Once the sequence was verified one more modification was made to the construct through the addition of the p3xFlags. This was done by ligation of the cathelicidin constructs in to the p3xFLAG-Myc-CMV-26 vector. *E. coli* were transformed and the colonies were selected for amplification and then purification of the plasmid occurred. H1299 cells were transfected via effectene lipofection methods, due to their high efficiency of transformation. A GFP-Flag-Apoptin control vector was used to determine if transfection occurred. Results showed the GFP vector being expressed when viewed at 200X magnification with a fluorescence microscope.

Materials

Table 1: List of Solutions and Concentrations for Protocols

Procedure	Solution	Concentrations
Nucleospin Plasmid Purification	Resuspension Buffer A1	50mM Glucose, 25mM TRIS-HCL and 10mM EDTA at pH8
	Lysis Buffer A2	100 µL NaOH (10N), 500 µL 10% SDS and 4.4. mL of water
	Neutralization Buffer A3	60mL 5M potassium acetate, 11.5mL glacial acetic acid, and 28.5 mL H2O
SDS-PAGE Gel Electrophoresis	Tris-Glycine Buffer	0.3 % tris, 1.44% glycine, and 0.1% SDS
	1x Tris buffered saline Tween-20	TBS-T 25mM Tris HCl, 137mM NaCl, 2.7 µM KCl, to 1000mL with ddH2O and 0.5% tween-20
	2X SDS-PAGE Loading Buffer	0.90 ml of 10% SDS, 0.45 ml of 100% Glycerol, 0.27 ml of 1M Tris, pH 6.8 0.63 ml of H_2O, 0.05 g of Bromophenol Blue, 0.25 ml of β-Mercaptoethanol
Bradford Assay	Low Detergent RIPA Buffer	Tris Base Lysis Buffer, NaCl, Glycerol, Triton X100 and Tween-20
	Protease Inhibitor Cocktail (PIC)	Leupeptin 18.8 mM, Pepstatin A 2.9 mM, Pefabloc SC 170 mM, Aprotinin 3.26 mM, Bestatin 6.5 mM

Methodology

Transformation of *E. coli* and Verification of Cathelicidin Construct

The pGEM vector with the recombinant cathelicidin DNA insert was amplified by *E. coli* transformation. Transformations were carried out with 50 µL of chemically competent *E. coli* and 2 µL of the pGEM plasmid with DNA insert in a cold Eppendorf tube. This mixture was incubated on ice for 20 minutes, heat shocked at 42ºC in a water bath for 45s, before it was returned to ice for another 2 minutes. The *E. coli* received 450 µL of prewarmed LB media and was incubated at 37ºC under shaking at 100 rpm for one hour. 50 µL of the transformed *E. coli* were plated on to Pre-warmed LB agar Ampicillin plates. These plates were incubated overnight at 37ºC.

The following day individual colonies were selected and inoculated in 3mL of LB with 100 µg/mL of Ampicillin media and incubated overnight at 37ºC with shaking at 190 rpm. Macherey and Nagel's Nucleospin plasmid purification protocol and kit (Cat. No. 740953) were used to isolate the plasmid. The 3mL of *E. coli* were centrifuged at 11,000 rcf for 30 minutes. The supernatant was removed and 250 µL of resuspension buffer A1 was used to resuspend the pellet. Then 250 µL of lysis buffer A2 was added to the tube and allowed to incubate at room temperature for five minutes. Then 300 µL of neutralization buffer A3 was added to the tube and then centrifuged at 11,000 rcf for 10 minutes. A column was added to a NoLid Eppendorf tube and 750 µL of the supernatant was added to the column and centrifuged at 11,000 rcf for 1 minute. 600 µL of wash buffer A4 (ethanol) was added and centrifuged at 11,000 rcf for 1 minute. The flow through was discarded and the silica membrane of the column was dried by centrifuging at 11,000 rcf for 2 minutes. The column was transferred to an Eppendorf tube with a top and 42 µL of water were added to the column and then centrifuged at 11,000 rcf for 1 minute. The column was discarded and the flow through in the Eppendorf tube contained the purified plasmid. The concentration of this plasmid collected was determined via a Nanodrop meter to measure its optical density.

To verify that the cathelicidin DNA was located within the pGEM vector collected in the miniprep, a restriction digest with 5µL plasmid DNA, 2µL 10x Buffer #2, 0.5 µL BamHI,0.5 µL EcoRI, 1 µL BSA, and 11 µL of ddH2O. The samples were mixed with the enzymes being added

last. The samples were centrifuged for 10s and incubated at 37ºC for one hour. An agarose gel electrophoresis (1%), pre-stained with 1 µL Ethidium Bromide, was run at 75V for 45 minutes. LL-37 fibronectin-BD was expected to run at 584 bp and LL-37 collagen -BD at 575 bp.

Cell Culture, Transfection, and Stable Cell Line Formation of H1299 Cells

Cell Culture

H1299 Cells were cultured in RPMI 1640 with 10% FBS. Cells were passaged every 72 hours or when 80% confluence was reached. During the splitting process cells and media were collected for testing for presence of protein of interest by Western blot analysis. Cells were rinsed with PBS prior to trypsinizing and placed in the incubator at 37ºC for one minute. Then, the plates were tapped gently to dislodge cells from the plate and examined under a microscope to ensure the cells had detached from the plates surface. The plate was then washed with fresh media and this media was collected in a conical tube. The tube was then centrifuged at 1000 rcf for five minutes to pellet the cells. The media was aspirated off of the pellet and the cells resuspended in fresh media. At this point a cell count of the resuspended media was done using a hemocytometer. In a 60 mm plate approximately 150,000 cells were plated in order to achieve 80% confluence in 72 hours. However, the LL-37 fibronectin-BD producing H1299 cells divide at a faster rate than the LL-37 collagen-BD producing H1299 cells and thus, about 100,000 cells of the LL-37 fibronectin –BD would be plated in order to achieve 80% confluence in 72 hours. If the pellet was resuspended in 5mL of media this would equal about 330µL of cell resuspension media being plated. Cells were incubated at 37ºC, high humidity, 19% O_2, and 5% CO_2 for 72 hours.

Transfection

Plasmid DNA from the previous step was used to transfect H1299 human lung carcinoma cells. H1299 cells were cultured in RPMI 1640 with 10% FBS in six well plates until the cells reached 80% confluence at 37^{o}C. Invitrogen's Lipofectamine (Cat. No.18324) was the reagent used during the transfection. 4.5 µg of plasmid DNA were added in to 250 µL of serum free media in an Eppendorff tube. In another set of tubes 250 µL of serum free media was mixed with 10 µL of lipofectamine. This was done in duplicate. Both tubes were incubated at room temperature for five minutes. The contents of each tube were combined together and incubated at room

temperature for 45 minutes. After 45 minutes the tube of combined solutions was added dropwise to the cells. Two wells received the LL-37 fibronectin-BD vector in lipofectamine and two wells received the LL-37 collagen-BD vector in lipofectamine, two wells were given no vector as the control. The wells were incubated overnight at 37ºC. Half of the wells were kept in order to be put under selection pressure using G418 to create a stable transfection. The other half, were used for Western blot analysis to confirm expression of protein of interest.

Stable Cell Line Generation

Invitrogen's G418 (Cat. No. 1013127) was used to kill cells that were not expressing the neomycin gene present in the recombinant peptide. 500 µg/ml of the G418 was added to the RPMI 1640 with 10% FBS media on the cells and allowed to incubate at 37ºC, high humidity, 19% O_2, and 5% CO_2 for 72 hours before the media was changed. After one week the cells were transferred from the 6 well dishes to 60mm dishes. Cells were passaged every three days in fresh media containing 500 µg/ml of the G418 until no cell death was present. At this point the G418 concentration was reduced to 100 µg/mL.

Localization of Cathelicidin Peptide

Protein expression and localization/secretion was determined by Western blot analysis. Whole cell lysate, soluble fraction, conditioned media and concentrated conditioned media were all tested for presence of protein of interest. Each sample was prepared in a different manner for Western blot analysis.

Sample Preparations

Conditioned media from untransfected cells was collected and used as a negative control. Conditioned media from both fibronectin-BD peptide transfected cells, and the collagen-BD peptide transfected cells, respectively was also collected and stored at 4ºC until used for Western blot analysis. 100µL of each sample was suspended in 100µL of 2X SDS-PAGE loading buffer and boiled for five minutes then stored at -20ºC until ready for analysis.

Trichloroacetic acid precipitation (TCA) protocol was followed in order to concentrate the conditioned media. 500µL of TCA (22%) was added to 1,000 µL of condition media. This was incubated at 4ºC for 10 minutes. The mixture was centrifuged at 14,000 rcf for five minutes. The

supernatant was removed and the pellet was washed twice with 500 µL ice cold acetone and centrifuged at 14,000 rcf for five minutes. The pellet was dried on a 95ºC heating block and 250 µL of 2X SDS-PAGE loading buffer was added to the dry pellet. The pellet was resuspended in the 2X buffer by breaking the pellet with the pipet tip and then suspending up and down several times. The solution was then boiled and stored at -20ºC until ready for analysis.

For the whole cell lysate, H1299 cells were trypsinized, washed with RPMI 1640 with 10% FBS media, centrifuged at 1,000 rcf for 5 minutes. Media was aspirated and the pellet was resuspended in 1,000µL of PBS and placed in an Ependorff tube. This was then centrifuged at 2,000 rcf for 10 minutes. The PBS was aspirated and the cells were suspended in 75µL of 2X SDS-PAGE loading buffer. The samples were then sonicated with the Ultrasonic Cell Disrupter for twenty pulses (Missonix XL-2000 series at power 3). The tubes were boiled for five minutes then stored at -20ºC until ready for analysis.

The soluble fraction samples were prepared by taking 200 µL of the whole cells suspended in lysis buffer and sonicating the cells with the Ultrasonic Cell Disrupter for twenty pulses at power 3. This was then centrifuged at 14,000 rcf for 15 minutes at 4ºC. 100µL of the soluble media was then transferred in to 100µL of 2X SDS-PAGE loading buffer and boiled for five minutes before being stored at -20ºC before western blot analysis.

SDS-PAGE gel electrophoresis and Immunoblotting

Western blot analysis was carried out using a 15% polyacrylamide resolving gel. The gel was poured using a Bio-rad MiniProtean Gel apparatus. Samples were denatured by boiling in 2X SDS-PAGE loading buffer for 5 minutes before loading. Different amounts of each sample (Table 2) were added per wells. 3 µL of Fermenta's Page Ruler Prestained Protein ladder (Cat. No. SM0671) was used as the standard.

Table 2: Loading Volumes

Whole Cell Samples	Soluble Fraction Samples	Conditioned Media Samples	Concentrated Conditioned Media Samples
5 µL	7 µL	20 µL	15 µL

The gel was run in a 1X Tris-Glycine pH 8.3 buffer at 160V for 60 minutes with a constant current. After running, the gel was transferred to a nitrocellulose membrane and run in a 1X Tris-Glycine transfer buffer (without SDS) containing 20% methanol for 1 hour at 100V constant current. After transfer, the membrane was immediately put on a shaker at room temperature for an hour in 5% non-fat dry milk in 1X Tris buffered saline Tween-20, pH 7.3. After, the membrane was incubated with anti-FLAG mAb M2 at 1/5000 dilution (Sigma, Cat. No. F3165-.2MG) in TBS-T overnight at 4ºC on a shaker. The membrane was washed 3 times for 10 minutes each in TBS-T. It was then incubated in rabbit anti-mouse at 1/5000 dilution (Abcam Cat. No. ab6729) for 1 hour on an agitator at room temperature. Then 3 washes for 10 minutes were applied again in TBS-T. Promega's Alkaline Phosphatase Western Blue Reagent (Cat. No. S3841) was used to visualize the bands on the membrane.

Determination of Protein Concentration in Samples

Bradford Assay

Whole cell lysate and soluble fraction were tested for total protein concentration. The whole cell lysate was prepared by resuspending the cell pellet in 200 µL of a low detergent RIPA buffer and sonicating the pellet with the Ultrasonic Cell Disrupter for twenty pulses at power 3. Samples were kept on ice until ready for analysis. For the soluble fraction samples were prepared by taking 200 µL of the whole cells suspended in RIPA buffer and sonicating the cells with the Ultrasonic Cell Disrupter for twenty pulses at power 3. This was then centrifuged at 14,000 rcf for 15 minutes at 4ºC. The supernatant was transferred to another Eppendorf tube and kept on ice until testing occurred.

In a 96 well plate 10 µL of low detergent RIPA buffer was put in to each well of the 96 well plate. Then 10 µL of BSA was added to the first well and serial diluted down the column dividing the concentration by a factor of two each time. This served as the control. In the other wells10 µL of sample was added to the first row and serial pipetted down the column increasing the dilution factor by two each time. 190 µL of Thermo Scientific's Coomassie Bradford reagent (Cat. No.23236) was added to each well as the colorimetric standard. Table 3 shows the dilutions layout for the 96 well plate. The optical density of the plate wells was measured through an automatic plate reader.

This process was repeated in later stages with the soluble fraction to be used in antimicrobial assays. To prepare the soluble fraction for testing 1 µL of PIC was added to the cell pellet. The pellet was then suspended in 200 µL of PBS and sonicated with the Ultrasonic Cell Disrupter for twenty pulses at power 3. This was then centrifuged at 14,000 rcf for 15 minutes at 4ºC. The supernatant was transferred to another Eppendorf tube and kept on ice until the Bradford assay was carried out.

Table 3: Dilutions Layout for 96-well plate in Bradford Assay

Fib-BD SF	Fib-BD SF	Col-BD SF	Col-BD SF	Fib-BD WC	Fib-BD WC	Col-BD WC	Col-BD WC	BSA	BSA
3	4	5	6	7	8	9	10	11	12
2	2	2	2	2	2	2	2	1	1
4	4	4	4	4	4	4	4	0.5	0.5
8	8	8	8	8	8	8	8	0.25	0.25
16	16	16	16	16	16	16	16	0.125	0.125
32	32	32	32	32	32	32	32	0.0625	0.0625
64	64	64	64	64	64	64	64	0.03125	0.03125
128	128	128	128	128	128	128	128	0.015625	0.015625
0	0	0	0	0	0	0	0	0	0

Antimicrobial Potency Testing of Recombinant Cathelicidin Peptides

G418 Resistant *E. coli* Growth Curve

In order to properly execute later testing, a growth curve for the *E. coli* strain being used was created. This was done by culturing the *E. coli* in identical conditions to which it would be cultured for later testing- 50mL in a flask, placed in an incubator/shaker at approximately 120 RPM at 37°C. The culture was done in LB Broth. At regular time intervals every 30 minutes, 3 mL of broth was taken from the culture and checked for optical density at 600 nm light

wavelength, then replaced. The growth was continued until it was past the logarithmic growth phase. At and OD_{600} of 0.925, dilutions of $1x10^4$, $1x10^6$, $1x10^7$, and $1x10^8$ were plated on LB Agar to determine the concentration of Colony Forming Units (CFUs). The plates were allowed to grow for 12 hours, and then colonies on the plates were counted visually via a light microscope.

Quantification of G418 Resistance of G418 Resistant *E. coli*

The concentration ranges of G418 and antibiotic solutions necessary to ensure proper negative controls for the live/dead assay were established by creating growth curves of the G418 resistant *E. coli.* The tests were done in a 96-well plate. Two fold dilutions were made starting from 250 µg/mL down to 7.8125 µg/mL. Each well contained 150 µL of LB Broth with antibiotic. The sample sets contained only G418. Six wells with no antibiotics were grown as well. All the wells were seeded with 1.5 µL of the G418 resistant *E. coli* grown for 16 hours in LB Broth, except for six wells which were filled with LB Broth as blanks. The whole plate was put into a 37°C incubator/shaker at 240 rpm, and optical density readings were collected by a plate reader starting after 2 hours of culture time. Further readings were made approximately every hour for 5 more hours. The exact set-up and concentrations are shown below in Table 4.

Table 4: Diagram of a portion of the 96-well plate setup for the G418 resistance test. Cells with a listed concentration of G418 also contained *E.coli*.

LB Broth	250 µg/mL	125 µg/mL	62.5 µg/mL	31.25 µg/mL	15.625 µg/mL	7.8125 µg/mL	0 µg/mL
LB Broth	250 µg/mL	125 µg/mL	62.5 µg/mL	31.25 µg/mL	15.625 µg/mL	7.8125 µg/mL	0 µg/mL
LB Broth	x	x	x	x	x	x	x
LB Broth	x	x	x	x	x	x	x
LB Broth	x	x	x	x	x	x	x
LB Broth	x	x	x	x	x	x	x

Antimicrobial activity of cathelicidin LL-37

Cathelicidin activity in soluble fraction

An assay using the soluble fraction from the stably transfected H1299 lysate was conducted. The soluble fraction was made in PBS as outlined previously for the Bradford assay procedure. Soluble fraction was generated by centrifugation at 14000 rcf for 15 minutes at 4ºC. The soluble fraction was then tested for any antimicrobial activity with the G418 resistant *E. coli*. The soluble fractions were diluted to achieve the same total protein concentrations in both samples, 3

mg/mL. Three concentrations of soluble fraction for exposure were created by keeping one sample of soluble fraction resuspended in 200µL PBS, one sample diluted in PBS to 1.5 mg/mL, and another sample diluted to 0.75 mg/mL. The total volume of all samples was 50 µL.

The *E. coli* was grown to an optical density at 600 nm light wavelength of 0.925, which corresponds to about $1.1x10^9$ CFUs/mL. The *E. coli* was diluted 100 fold in LB Broth, and then 100 µL was added to the soluble fractions. The exposure lasted 16 hours. The bacterial cells were stained using Invitrogen Live/Dead Baclight© stains, with 0.3µL of each dye added to each sample. Pictures were then taken using a Nikon AZ100 microscope with 40x objective and fluorescence, and were analyzed using ImageJ. The counts were done by first creating a color threshold, which made the bacteria in the image more distinct. The images were then made binary, eliminating all color and reducing them to black/white. The particle detection software then gave bacterial counts. Some counts were checked by visual inspection to ensure the accuracy of program settings.

Collagen Film Development in a Quartz Crystal Micrograph with Dissipation Monitoring (QCM-d)

The QCM-d was prepared using a washing procedure as follows: 10 mL of DI water, 0.01M SDS, and 10% ethanol were flowed through the chambers at 0.3 mL/min. The crystals were then removed from the chambers, dried off using a high flow of Nitrogen gas, and plasma etched. Afterwards they were placed back into the QCM-d. PBS was then flowed through at 0.1 mL/min until a stable baseline reading was achieved. After 5 minutes of stable baseline reading, 10mL of collagen at a concentration of 0.5mg/mL was flowed through the chambers at a flow rate of 0.1mL/min. After the collagen flow, PBS was flowed at 0.1 mL/min to test the durability of the film that had attached to the crystals. After 3 hours, the film stabilized again and the flow rate of PBS was increased to 0.3 mL/min to test the resistance to shear forces.

Results

Verification of the Cathelicidin Construct Plasmid DNA

Agarose gel electrophoresis analysis of the restriction digest with EcoRI and bamHI, shows presence of LL-3 Collagen-BD and LL-37 Fibronectin-BD in the pGEM vector (Figure 5). Data shows that the digested samples have fragments produced at approximately 600 base pairs. The predicted size of the LL-37 DNA is 575 and 584 base pairs for collagen binding domain and fibronectin binding domain respectively.

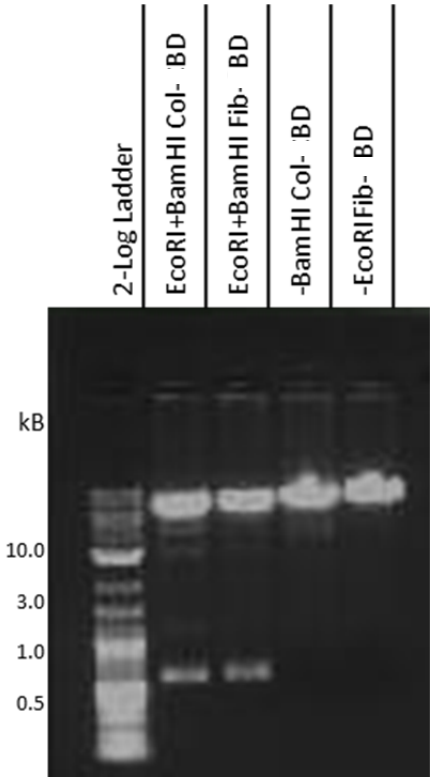

Figure 5: Restriction Digestion of pGEM Vector showing plasmid DNA inserts

Stable transfection

Initially, approximately 300,000 cells were plated after transfection in to the six well dish for the fibronectin-BD Cathelicidin H1299 cells, collagen-BD Cathelicidin H1299 cells, and the untransfected H1299 cells. Half of the cultures were used in Western blot analysis for expression and the other half were put under selection pressure with 500 µg/mL of G418. After six days all of the cells in the untransfected H1299 cell culture had died in the six well dish. After six days, the fibronectin-BD Cathelicidin H1299 cells were at 80% confluence and the collagen-BD Cathelicidin H1299 cells were at 65% confluence. All cells were collected and the cells were moved to 60 mm dishes. Cells were split twice more after 72 hours each until no cell death

appeared in the 60 mm dishes and then they were passaged to a 10 cm dish. Cells were allowed to grow for six days and the media was changed every 72 hours. At this point stocks were frozen in liquid nitrogen. The cells were moved back to a 60 mm dish in order to continue collection of conditioned media and the G418 concentration was reduced to 100 µg/mL. Cells were split for three more weeks every 72 hours until the cell lines were terminated.

Western Blot Analysis of Transfected Cells

In order to see if the transfected H1299 cells were expressing the recombinant Cathelicidin peptide, a Western blot analysis was run. Western blot analysis allows for the visualization of the expression of the peptide. Taking samples from different aspects of the cell culture such as the conditioned media, the whole cell lysate and the soluble fraction of the whole cell lysate also allowed for a determination of where the peptide was expressing strongest. Predicted molecular weight of the protein by Western blot was expected at approximately 22 kDa. Figure 6a shows the Western blot of the collagen binding domain Cathelicidin transfected H1299 cells expressing the peptide in the whole cell lysate, soluble fraction, and conditioned media. Furthermore, the concentrated conditioned media shows expression of the peptide however; the expression is at a lower molecular weight. Figure 6b shows the Western blot results from the fibronectin binding domain Cathelicidin transfected H1299 cells. The peptide is expressed in both the whole cell lysate and soluble fraction. However, unlike the collagen binding domain Cathelicidin the concentration of peptide in the conditioned media for the fibronectin binding domain was below the sensitivity of the Western blot at 2ng and no expression was seen. However, peptide is being secreted as the concentrated conditioned media sample shows expression. Again, this expression is at a lower molecular weight than the peptide being expressed by the whole cell and soluble fraction. For both Western blots there was no expression in the untransfected H1299 cells.

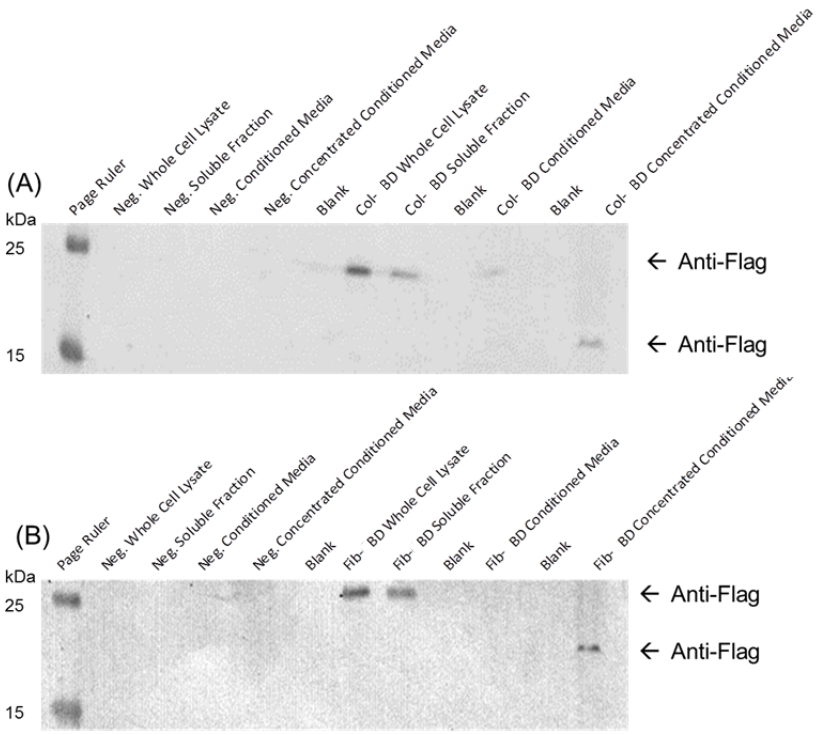

Figure 6: Western Blot analysis of the Transfected H1299 Cells (A) Western Blot analysis of the negative control samples and collagen binding domain cathelicidin peptide transfected H1299 cells. (B) Western Blot analysis of the negative control and fibronectin binding domain cathelicidin peptide transfected H1299 cells.

Bradford Assay Results

A Bradford assay was carried out to determine to the total protein concentration in the samples loaded in the Western blot. Table 4 shows the concentration of total protein present in the whole cell lysate and soluble fraction samples for both binding domains of the recombinant Cathelicidin peptide loaded in to the Western blots pictured in Figure 8. Because the recombinant Cathelicidin peptide was not purified these concentrations in Table 4 are for all cellular protein in the samples of which the desired protein is a small fraction.

Table 4: Concentration of Peptide in Soluble Fraction and Whole Cell Lysate in Western Blot Samples

Samples	Protein Concentration
Collagen-BD Whole Cell Lysate	8.85 μg/μL
Collagen-BD Soluble Fraction	7.8 μg/μL
Fibronectin-BD Whole Cell Lysate	10.5 μg/μL
Fibronectin-BD Soluble Fraction	8.0 μg/μL

E. coli growth curve and Cell Concentration

All of the antimicrobial assays were conducted with a strain of *E. coli* which is resistant to Neomycin, Kanamycin, and G418. This was necessary as the condition media containing the recombinant peptides also contained G418, which was used for selection of positively transfected cells. The antimicrobial testing requires cells to be in a particular growth state that has a known cellular concentration and has few if any dead cells present. This state was found by plotting a curve of optical density against growth time, called a growth curve, which is show Figure 7 below.

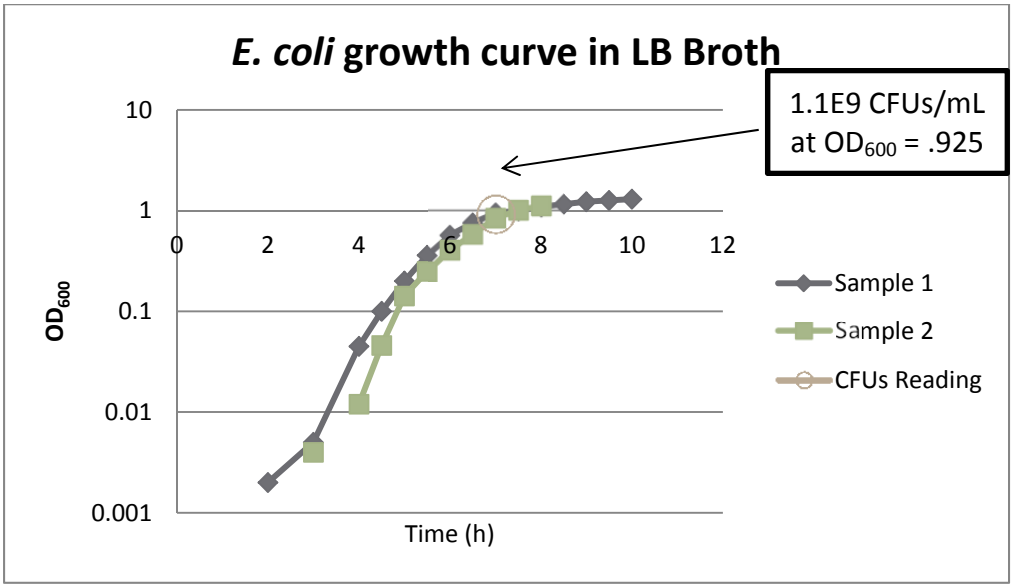

Figure 7: *E. coli* growth curve. Plot of Optical Density at 600 nm wavelength versus time in hours. CFUs/ mL result shown at tested point. Log scale for Optical Density. Two trials with identical conditions, grown in LB Broth.

After an ideal growth time and corresponding optical density was found, dilutions of the growing cell solution ranging from $1x10^4$ to $1x10^8$ were plated and allowed to grow. The number of colonies on each plate was counted and converted into a 'colony forming unit' (CFU) concentration in the solution. A CFU is typically not a single cell, but a small cluster which can survive some stress and continue to reproduce. The results are shown in Table 5 below.

Table 5: *E.coli* colony growths when plated at dilutions from $1x10^4$ to $1x10^8$. Averaged result of CFU concentration calculations suggests typical cell concentration at selected growth state measured by optical density.

Dilution	Colony Count A	Colony Count B	Average	CFUs per mL Solution
$1x10^4$	Thousands	Thousands	N/A	N/A
$1x10^6$	~800	~900	~850	$8.50x10^8$
$1x10^7$	115	119	117	$1.17x10^9$
$1x10^8$	12	15	13	$1.30\ x10^9$
Average CFUs/mL:				$1.11\ x10^9$

Quantification of G418 Resistance

The results of the growth curve test for G418 resistance are shown below. G418 resistance results indicated whether testing could be conducted with conditioned media, which contained high concentrations of G418. The results are shown below in Figure 8.

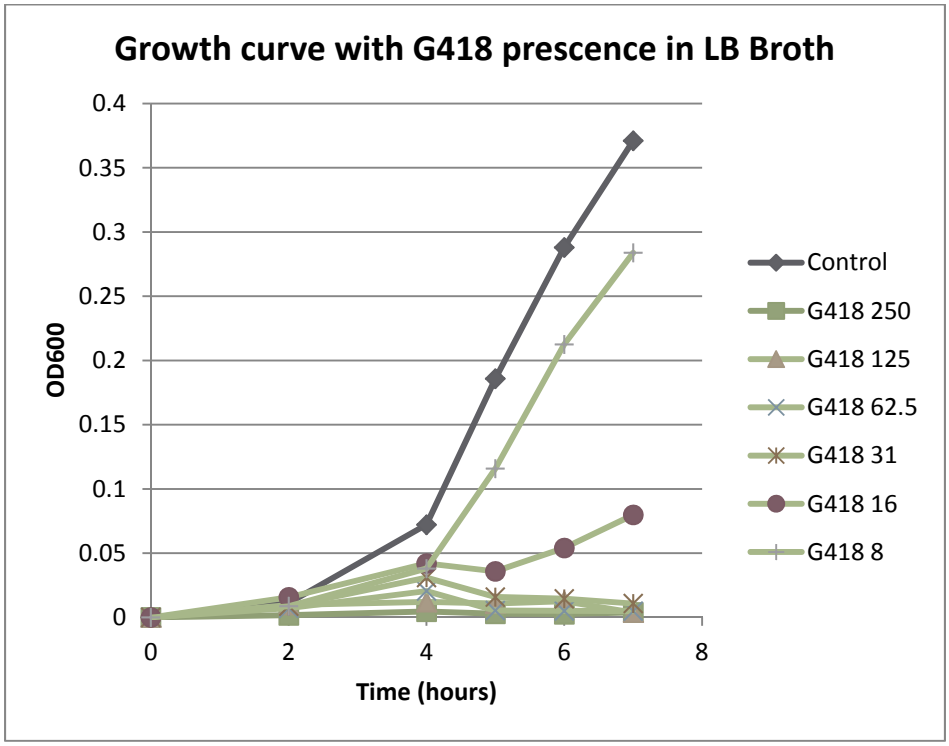

Figure 8: Optical Density at 600nm wavelength versus time for serial dilutions of G418 in LB Broth. Graph shows effect of increasing G418 concentration on growth of G418 resistant *E.coli*. Averages of two trials, standard deviations not shown to allow clarity at low OD600 values.

The growth curves show that the growth of the G418 resistant *E.coli* is being almost totally suppressed with G418 concentrations as low as 31 µg/mL. This is much lower than the 100µg/mL present in the conditioned media. Further testing required soluble fractions from stably transfected H1299 cells.

Soluble Fraction Antimicrobial Assay

This assay shows the antimicrobial effect of the recombinant peptides on G418 resistant *E.coli* after exposure from 12 to 16 hours. An initial sample was diluted 1:2 and 1:4 with PBS, and the results for all the concentrations were very similar. Two trials were run, and the average results were graphed along with standard deviation. A graph of live bacteria count (**Error! Reference source not found.**9) at the highest total protein concentration is shown to demonstrate any suppression of bacterial growth, and graph of kill percentage (Figure 10) at the highest total protein concentration is shown to demonstrate any killing of bacteria compared to negative control.

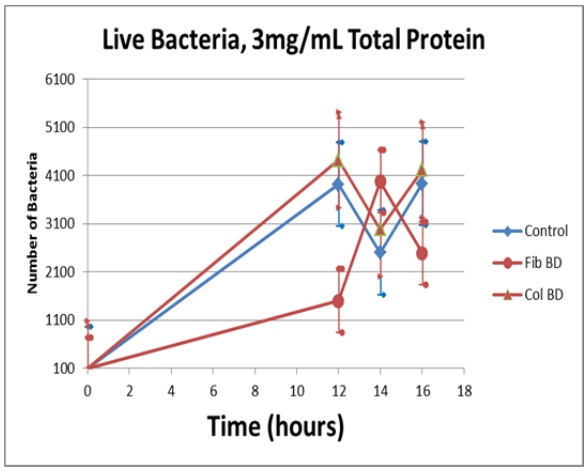

Figure 9: Live bacterial counts from soluble fraction assay with G418 resistant E.coli. N=2. Error bars show standard deviation of data sample. Total protein concentration 3 mg/mL.

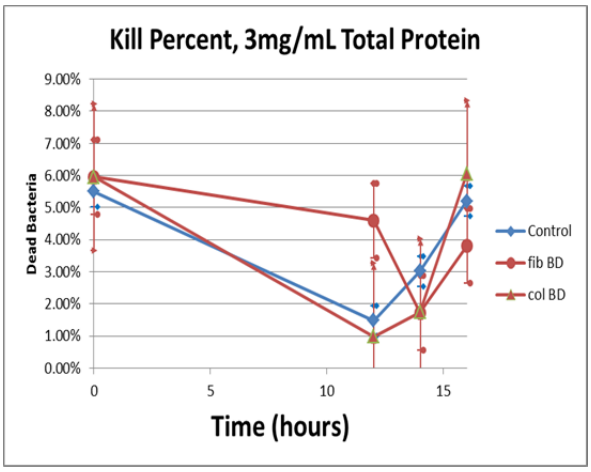

Figure 10: Dead bacteria percentages from soluble fraction assay with G418 resistant E.coli. N=2. Error bars show standard deviation of data sample. Total protein concentration 3 mg/mL.

The live bacterial counts do not show any suppression of growth; the bacteria counts are all in a similar range. The kill percentage drops during the exposure, as bacterial growth outpaced bacterial death during the exposure. Again, the data points are all in a similar range. The recombinant peptides do not appear to be killing the *E.coli* or suppressing growth at these

concentrations and experimental conditions. The results of the lower total protein concentration samples are similar, and can be seen below in figure 11.

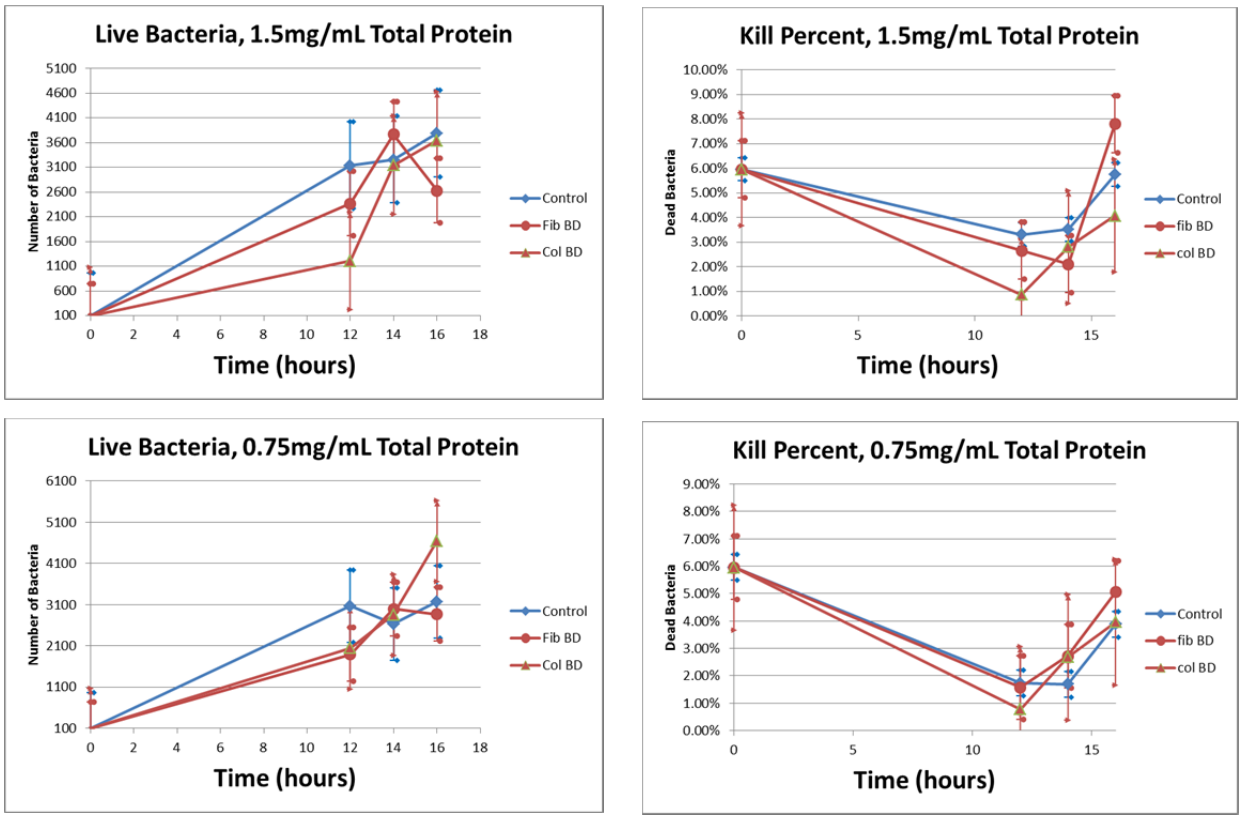

Figure 11: Live G418 Resistant *E.coli* bacteria counts and dead cell percentages for lower total protein concentration samples. N=2. Error bars show standard deviations. 1:2 and 1:4 dilutions of original soluble fraction samples, all other conditions identical.

Collagen Film Development in QCM-d

A collagen film was deposited onto the QCM-d crystal surfaces and tested to observe whether a stable film could be developed, and if so the resilience of the film. Results are shown below in Figure 12.

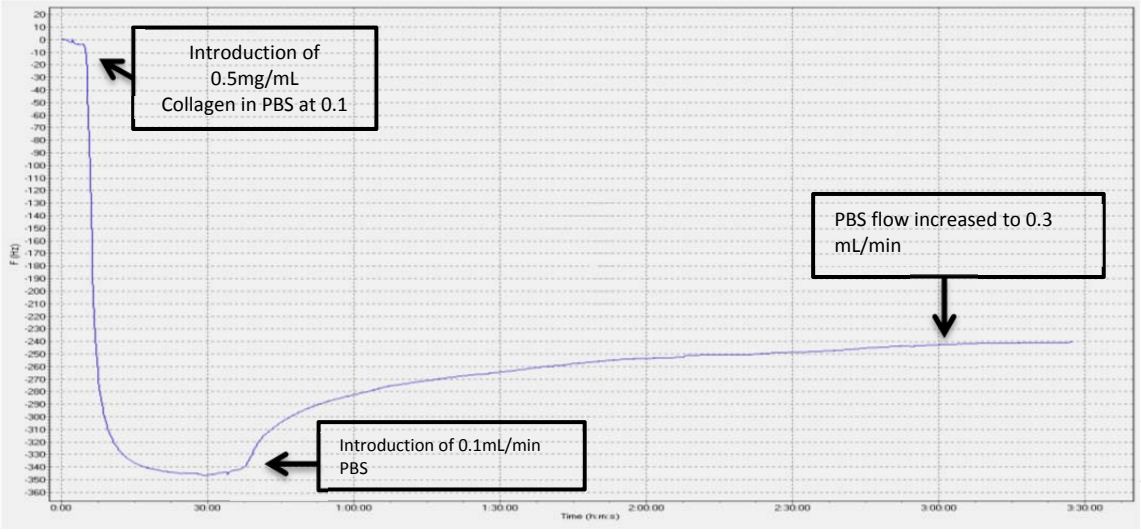

Figure 12: Collagen film development in QCM-d. Vertical Axis in Hz differential, horizontal axis is time. Collagen film rapidly deposited, degraded slightly over three hours of PBS flow, and then stabilized.

Quickly after the collagen solution begins flowing through the chambers, a film begins to attach to the crystals. When PBS was flowed through the chambers, the collagen film degraded somewhat over a 3 hour period, and then stabilized. When the flow rate of PBS was tripled, no further loss of film was observed. This suggests the film is resilient to some shear strain and reliable enough for further attachment testing with the purified recombinant peptides.

Discussion

The long-term goal of this research is to produce a cell-derived ECM with the continuous production and secretion of the recombinant forms of human Cathelicidin LL-37. This would be implemented for the treatment of chronic dermal wounds, in particular those infected by multiple-drug-resistant strains of bacteria. The goals of this last year of research were to verify the presence of the plasmid DNA insert from the previous research, to stably produce the recombinant Cathelicidin LL-37 forms in H1299 cells, to determine localization of the peptides, to conduct antimicrobial testing in solution, to conduct attachment testing to collagen and fibronectin films, and to conduct antimicrobial testing of the peptides after attachment.

Restriction-digestion with EcoRI and BamHI confirmed the presence of the insert in the pGEM vector. LL-37-fibronectin BD and LL-37-collagen BD migrate at the predicted size on 1% agarose gel electrophoresis. The next goal that was achieved was the transfection of H1299 cells and the creation of two stable cell lines, one expressing each recombinant peptide. The generation of these lines took approximately two weeks with passaging every 72 hours at this point no cell death was observed.

The localization of the peptide expression was investigated using Western blot analysis with anti-FLAG antibody. The expression was first observed in the whole cell lysate for both recombinant peptide forms, confirming that expression was occurring. Furthermore, expression also occurred in the soluble fraction for both peptide forms. For these samples, expression occurred at the expected molecular weight of 22 kDa.

Expression of the peptide was seen in the conditioned media for the collagen binding domain peptide form, but not for the fibronectin binding domain peptide form. The concentration of the fibronectin form peptide appears to be below the sensitivity threshold of 2 ng of protein for the Western blot. To try to detect the peptide, conditioned media samples were concentrated via TCA precipitation. The Western blot analysis of the concentrated conditioned media shows expression for both recombinant peptide forms, but at a lower molecular weight than expected. This could be as a result of recombinant peptides being degraded by the TCA precipitation

process, causing cleavage of approximately 5 kDa, or the recombinant peptides are being cleaved by the H1299 cells during the secretion process.

Even though expression was seen at the expected molecular weight in the collagen-BD recombinant peptide conditioned media, this does not necessarily indicate that some of the peptides are not being cleaved through the secretion process. The amount of cleaved peptide present in the conditioned media could have been less than the sensitivity of the Western blot assay. Because there is no detection of full length peptides in the fibronectin-BD conditioned media, it is impossible to make a conclusion at this point regarding processing of LL-37 fibronectin BD. The cells could be cleaving off most of the peptide in the secretion process, or all the cleaving could be from the TCA precipitation. To fully understand the secretion and processing of the recombinant peptides more experimentation is required.

The conditioned media contained G418, the antibiotic used to create a stable cell line by selecting for neomycin resistance that the transfected cells possessed. Because of this, any antimicrobial testing on the recombinant peptides that had been secreted into the conditioned media would have to overcome the presence of G418. A strain of G418 resistant *E.coli* was tested for the extent of its resistance, and was found to be not suitable at the concentrations of G418 present in the conditioned media. The G418 concentration growth curves showed that *E. coli* growth was being entirely suppressed at a G418 concentration of 62.5 µg/mL, lower than the 100 µg/mL present in the conditioned media of the stable cell lines. As a result of this, antimicrobial testing had to be conducted with the soluble fraction from the whole cell pellets, which did not contain G418, but which was a more limited supply.

The antimicrobial testing showed that, at the concentrations of peptide present, and the conditions that were tested, the recombinant peptides are not suppressing growth or killing the G418 resistant *E. coli.* However it cannot be concluded from these results that the peptide has no antimicrobial effect. Testing needs to be conducted at more temperatures, on other buffer systems, for longer exposure durations, and on other bacteria strains in order to form conclusions about the activity of the recombinant peptide forms. Furthermore, the peptides need to be purified to get accurate measurements of the concentrations present during antimicrobial testing.

Another concern is that the recombinant peptide forms may not be active until after being secreted by the cells. A common aspect of the peptide secretion process is that the peptides are folded and cleaved in a way that typically causes a conformational change into the active form. This could mean that the soluble fraction that was tested for antimicrobial activity contained only inactive form of the peptides as the peptide was isolated from inside of the cells. Further research comparing the activity of secreted and unsecreted peptide would be required to investigate this possibility

The attachment testing was limited in scope, consisting only of demonstrating a collagen film developed onto a silicone crystal surface using a QCM-d. The collagen film developed at low concentrations of collagen and was durable enough for the potential testing of attachment of the collagen-binding recombinant peptide. Fibronectin film development was not tested as it was decided at the time that focus on the antimicrobial testing was more important. However, attachment testing with the soluble fraction or conditioned media was not possible. This is because the majority of the protein in these solutions is not the recombinant Cathelicidin LL-37 peptides, and these other proteins would cause too much noise in the readings to be able to detect the influence of the recombinant Cathelicidin peptides. Because of this, purified peptides are necessary for further attachment testing. Moreover, as attachment testing of the peptides could not be conducted the antimicrobial testing after attachment could not be done.

This research showed some definite conclusions. The presence of the insert was confirmed in the pGEM vector. Two stable cell lines which expressed each recombinant peptide were created. The recombinant peptide was present both in the whole cell and in the conditioned media for both recombinant peptides. The peptide forms are not demonstrating antimicrobial activity and do not show suppression of *E. coli* growth under these antimicrobial assay conditions. Finally, a collagen film was developed in the QCM-d and shown to be durable enough for future attachment testing.

For further research in the immediate future, the first concern is to purify the recombinant peptides. This can be done using affinity chromatography with an anti-FLAG resin. This would allow more precise antimicrobial testing and attachment testing onto collagen and fibronectin films in the QCM-d. The antimicrobial testing needs to be expanded based on published research showing antimicrobial activity of wild-type Cathelicidin LL-37 in order to form any conclusions

on the activity of the recombinant peptides. Alternative concentration methods could be employed to increase concentration of recombinant peptide in the conditioned media. This will allow for the determination of if the current TCA precipitation is a suitable method of concentration. As well, antimicrobial activity after attachment to collagen and fibronectin films needs to be conducted which will require the purification of the recombinant peptides.

To achieve the long-term goal of a cell-derived ECM with an integrated AMP as a treatment for chronic dermal wounds, this research still has far to go. However, if this could be produced, treatment for chronic dermal wounds would be vastly improved. The cell-derived ECM with integrated recombinant Cathelicidin LL-37 would facilitate healing, prevent further infection from drug-resistant bacterial strains, and stimulate the human immune response to the wound.

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